

Factors affecting the development of *Phytophthora alni* ssp. *alni* infections in *Alnus glutinosa* L.

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ABSTRACT: *Phytophthora alni* is responsible for a devastating disease in alder and changes in the environment of riparian and alder carr ecosystems. One of the main approaches to solve this problem is to find naturally resistant genotypes using a series of artificial inoculation experiments, to preserve and use them in programmes for resistance breeding. However, the results of artificial inoculation experiments (screening for natural resistance) can be affected by several factors. The potential effect of the social status of the host, the presence of naturally occurring *P. alni* infections, the season and the size of the sections of branches used were studied in a series of infection experiments. It was found out that the development of lesions was significantly affected by the year season (the largest lesions were found in summer) and by the presence of naturally occurring *P. alni* infections in the sampled trees (the lesions were five times larger in healthy trees and trees recovered from natural *P. alni* infections compared to trees with active disease development).

Keywords: *Alnus glutinosa*; black alder; *Phytophthora alni* subsp. *alni*; infection experiment; natural infection; seasonal variation

An alder tree disease caused by the oomycete fungal pathogen, *Phytophthora alni*, was first observed on the banks of streams and rivers in Britain in 1993 (GIBBS 1995). Since then, the disease has been reported in many other European countries (e.g. GIBBS et al. 1999; SZABÓ et al. 2000; STREITO et al. 2002; JUNG, BLASCHKE 2004; OSZAKO, ORLIKOWSKI 2005) and has caused a devastating epidemic in alders. In the Czech Republic, the pathogen was first isolated from damaged black alder trees in Western Bohemia in 2001 (ČERNÝ et al. 2003) but was properly identified only later (ČERNÝ et al. 2008). To date, the pathogen has been found in approximately 150 sites, although the disease symptoms have been detected in approximately 300 sites throughout the Czech Republic. At present, the pathogen most likely occurs along

thousands of kilometres of river banks in the Czech Republic and causes significant tree losses; as a result, it has become a major threat to riparian and alder carr ecosystems. In particular, the impact of the pathogen can be seen in changes in the structure and stability of riparian alder stands and in local changes in the stability of river banks (ČERNÝ, STRNADOVÁ 2010). The screening of native alder populations for naturally occurring resistance (in a series of artificial infection experiments), the preservation of more resistant genotypes and the subsequent breeding and broad use of these resistant genotypes are possible ways of managing the disease.

The idea of natural resistance to *P. alni* occurring in *A. glutinosa* has been suggested by the fact that healthy mature alder trees can be found in the same

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stand as both diseased and dead trees (STREITO et al. 2002; JUNG, BLASCHKE 2004). Furthermore, the occurrence of different levels of resistance was observed by JUNG and BLASCHKE (2006), who reported a high intraspecific variation in the susceptibility of black alder to *P. alni* ssp. *alni* during a series of infection experiments. Therefore, we initiated a screening programme with the aim of finding resistant alder trees that could be used in breeding programmes; the potential value and success of such breeding programmes were demonstrated by the work on *Chamaecyparis lawsoniana* and its pathogen, *Phytophthora lateralis* (e.g. SNIETKO, HANSEN 2003).

Before starting such a screening programme, it is very useful to have the good knowledge of factors that potentially influence the development of artificial infections, to avoid erroneous and potentially misleading results in the selection process. BURGESS et al. (1999) showed that flooding and subsequent hypoxia was a very important factor leading to higher levels of damage in the host by *Phytophthora* infections and the strong effect of water stress on *P. alni* infections in black alder has been clearly demonstrated (STRNADOVÁ et al. 2010). Thus, the trees suffering from a high water table and flooding should be excluded from the screening. However, the potential effect of other factors has been studied to a lesser extent. The effect of the season on *Phytophthora* disease development can be expected on the basis of experiments carried out with red oak, black alder and common beech (ROBIN et al. 1994; BRASIER, KIRK 2001; HOLUB et al. 2010). Furthermore, JUNG and BLASCHKE (2006) suggested the potentially confusing effects of naturally occurring (former and current) *P. alni* infections in artificial infection tests. There are also other factors that might possibly influence the results of infection experiments and should, therefore, be taken into account. The first factor is a possible effect of the social status of the individual trees (well-developed, dominant trees in sunny locations may be more resistant than poorly developed, suppressed trees suffering from competition). The second factor is the duration of the infection experiment (the changes in the growth rate of necrosis during the infection experiment can potentially influence the final evaluation). The last factor is the size (thickness) of the branches being sampled (a factor potentially influencing the aging of the host material during the artificial inoculation test).

The aim of this study was to evaluate the potential effect of the factors of social status and health status of the sampled tree, effect of the season, duration of the experiment and size of the tested

sections of branches on the outcomes of artificial infection experiments.

MATERIAL AND METHODS

Plant material and treatments

Four adult black alder (*Alnus glutinosa*) trees from the area of Česká Sibiř (Ješetice, Central Bohemia, coordinates 49°35'21.83"N, 14°35'37.26"E) were chosen for infection experiments. The trees were nearly of the same age and size and were situated in the same stand on the alluvial plain of the Mastník River in the Vltava River Basin. Potential effects of the five aforementioned factors on artificial infections were studied in two experiments carried out in 2009 and 2010.

The first experiment examined possible effects of the social status, as determined by the trunk position in a multi-trunk tree. Dominant well-developed trees at sunny places have a higher amount of foliage and thus can store a higher amount of assimilates into the sink than underdeveloped trees. The assimilates can be used later as a source of specific metabolites and energy against the pathogen during the invasion (cf. PROCHÁZKA et al. 1998). Thus the well-developed trunks inside the multi-trunk tree can be more stress-tolerant than the suppressed ones. Therefore, samples were collected in July 2009 from two equally aged (approximately 25 years old) stems belonging to just one multi-trunked black alder tree without any signs of *Phytophthora alni* infections. The stems differed in their position on the multi-trunk tree (the centre and southwest margin, respectively) and, thus, in their level of insolation and competition. The first, highly shaded and poorly developed stem, denoted as suppressed, was located in the centre of the multi-trunk tree. The stem was thin (~ 10 cm at diameter at breast height – DBH), with a high proportion of withered branches in the lower part of its crown. The crown had sparse foliage, with no indications of flowers or fruits, and had apparently suffered from competition with other stems in the multi-trunked tree and low light levels. The second stem, denoted as dominant, fully exposed to the sun, was located on the southwest margin of the multi-trunked tree. It was well developed (~ 20 cm at DBH), without dead branches, and had dense green foliage (without any indication of yellowing or damage) and well-developed fruits. The design of the experiment also enabled to evaluate the effect of the duration of the experiment on lesion development; the diameters of the lesions

were measured in one, two and three weeks following the inoculation.

In the second experiment, the effect of naturally occurring *P. alni* infections and the possible influence of the year season were investigated using sections of branches obtained from three black alder trees (different from the multi-trunk tree in the first experiment) of different health state. The first tree (denoted as healthy) did not exhibit any signs of natural *P. alni* infection on the collar, buttresses or foliage (thinning, yellowing). The second sampled tree (denoted as diseased) was acutely infected with *P. alni* and showed actively bleeding cankers from which the pathogen was isolated. The foliage of this tree (the second) was significantly thinner, and the leaves were small, yellowish, and sparse. The third tree (denoted as healed) apparently recovered from an earlier *P. alni* infection, when it was approximately 2 or 3 year old, and a tongue-shaped necrotic area, apparently caused by *P. alni*, was found at the base of this tree. The necrotic tissues were typically black and rotten and were separated from the surrounding healthy tissue by callus formation and an attempt to isolate the pathogen from this tree (the third) was unsuccessful. In addition, the foliage of the third tree showed marks of recovering from the disease: the crown was reduced, but the foliage was well developed, green and only partially reduced in size. Two or three branches from each of these three trees were collected at four different times, in August and October of 2009 and in March and June of 2010.

The observations from the first (one week after inoculation) and the second (healthy tree; all sampling dates) experiments were also used to evaluate the potential effect of the diameter of the tested sections of branches on lesion development.

Inoculation and incubation

At least 20 replicates of the sections were prepared from the collected branches of the above-mentioned stems and trees in each treatment of the two experiments (social status \times weeks after inoculation in the first experiment and health state \times season in the second experiment). The sections of branches were straight, non-branching, and approximately 10 cm long and 1.5–4.0 cm thick. All the sections were numbered, and their diameters were measured at the centre in two directions at 90° angles to each other.

First, the middle of each section was surface sterilized with 97% ethanol. Using a flamed cork borer,

a hole 6 mm in diameter was then made in the bark of this sterilized area to expose the phloem. The bark plug was removed, and the section of branches was inoculated by replacing it with a V8A agar plug (ERWIN, RIBEIRO 1996) taken from the margin of a 7-day-old actively growing colony of a fresh isolate of *P. alni* ssp. *alni*, with the aerial mycelium facing the wood. This culture, No. P271.09, is preserved in the culture collection of our institute (RILOG). The control sections were prepared in the same way and were inoculated with sterile V8A agar plugs. The wounds were covered with Parafilm®, and the sections placed individually, distal end down, into flasks containing deionized water to a depth of approximately 2 cm. The flasks were then placed in an incubator at 20°C and protected from light. In the first experiment, the sections were incubated for one, two, and three weeks in July 2009. In the second experiment, the sections were incubated for one week, and the experiment was repeated four times, in August and October of 2009 and in March and June of 2010. The treatments in both experiments were totally randomized.

Measurement

In both experiments, we measured the length and width of each lesion appearing on the sections of branches. The necrotized phloem was exposed by carefully removing the periderm with a scalpel. The border between the healthy bright-coloured tissues and infected red-coloured tissues was easily visible, and the tissues were usually separated by a thin but well-visible black line, as described in DAVIDSON et al. (2003). The maximum length and width of each lesion were measured in two directions at right angles to each other. The size of the hole made in the phloem (6 mm) was then subtracted from the measurements. In the second experiment, the necrotic area of the phloem was outlined and the outlines were then traced onto tracing paper and digitized. The surface area of each lesion was calculated using the ImageJ 1.43 software (NIH, Bethesda, USA).

Data analysis

The data were analyzed using STATISTICA 8.0 software (StatSoft Inc., Tulsa, USA). Prior to the analysis, the data were tested for normality and homogeneity; even though the data were transformed using common logarithms, ANOVA was

then judged to be an inappropriate method. The Kruskal-Wallis test was, therefore, chosen to analyze the differences in the length, width and area of the lesions in each of the data sets. The multiple comparisons of the mean ranks for all the groups were computed when a statistically significant difference was found. The social status (as defined by the position in the multi-trunked tree), health state (presence or absence of natural *P. alni* infection), and the year season of sampling were selected as the fixed factors, and the length, width and area of the lesions were selected as the dependent variables. The correlation between the data in the first experiment was tested using the Spearman rank correlation test. This test was also used to determine the correlation between the thickness of the branch section and the length, width and surface area of the lesions. For this purpose, we only used the data obtained from the “dominant” stem in the first experiment and from the “healthy” tree in the second experiment.

RESULTS

A potential effect of the social status of the sampled stem on the artificial infection development was not confirmed during the experiment. No statistically significant differences were found after inoculation for the lengths and widths of lesions developed in the suppressed or dominant stems for any of the three incubation times (one, two, and

three weeks after incubation), with the exception of the second time period when only slightly significant differences between the lesion lengths were observed (Table 1).

The comparison of artificial infection development after one, two, and three weeks after inoculation showed that the lesion width and length developed almost linearly during the three weeks. The measurements at each time period were significantly correlated with each other ($r = 0.443-0.670$, $P < 0.05$) except for the data on the width and length in the first and third periods. Moreover, the infection developed rapidly from the beginning of the experiment, and after the first week, the average lesion diameter was 65.31×16.27 cm for the pooled data of the dominant and suppressed trunks.

The investigation of the effect of the host’s health state on the development of artificial infection revealed that natural infections of *P. alni* had a high impact on the development. The average values of the three characteristics of lesions (width, length and surface area) in the sections of branches obtained from the diseased tree (i.e. acutely infected with *P. alni*) differed from those of the sections of branches obtained from both the healthy and healed trees in August 2009 (Table 2, Fig. 1). The average values for the lesions in the material collected from the acutely infected trees were nearly five times lower than the others (the lesion surface area was 115 mm^2 in comparison with 497 and 552 mm^2 of the healthy and healed trees, respectively). In June 2010, statistically significant differ-

Table 1. Effect of social status of sampled stem (suppressed and dominant), and duration of experiment on extent of necrose caused by of *P. alni* in black alder

Social status	Lesion width (mm)					
	1 st week		2 nd week		3 rd week	
	<i>n</i>	mean (\pm SE)	<i>n</i>	mean (\pm SE)	<i>n</i>	mean (\pm SE)
Suppressed	26	16.04 (\pm 1.32) ^a	24	29.42 (\pm 2.99) ^a	24	49.00 (\pm 3.46) ^a
Dominant	23	16.52 (\pm 1.16) ^a	22	23.64 (\pm 2.16) ^a	22	41.18 (\pm 3.72) ^a
Social status	Lesion length (mm)					
	1 st week		2 nd week		3 rd week	
	<i>n</i>	mean (\pm SE)	<i>n</i>	mean (\pm SE)	<i>n</i>	mean (\pm SE)
Suppressed	26	62.54 (\pm 4.24) ^a	24	87.00 (\pm 6.55) ^a	24	156.67 (\pm 15.66) ^a
Dominant	23	68.43 (\pm 2.27) ^a	23	114.13 (\pm 10.27) ^b	23	180.65 (\pm 12.63) ^a

Social status: suppressed – poorly developed stem (due to low input of radiation and competition of other stems), dominant – well developed stem in the margin of multi-trunk tree suppressing neighbouring stems, *n* – number of observations (infected segments), values – mean and standard errors (SE) – followed by the same index are not statistically different ($P > 0.05$) between treatments

Table 2. Effect of health status (natural infection of *P. alni* in sampled trees) and time of year on artificial infection of *P. alni* in black alder (after one week of incubation)

Treatment	Lesion width (mm)		Lesion length (mm)		Surface area of lesion (mm ²)	
	<i>n</i>	mean (± SE)	<i>n</i>	mean (± SE)	<i>n</i>	mean (± SE)
April						
Healthy	33	2.91 (± 0.21) ^a	33	3.30 (± 0.34) ^a	33	51.35 (± 3.74) ^a
Diseased	35	2.51 (± 0.15) ^a	35	2.69 (± 0.16) ^a	35	42.02 (± 2.10) ^a
Healed	37	2.49 (± 0.14) ^a	37	2.65 (± 0.14) ^a	37	42.86 (± 1.88) ^a
June						
Healthy	26	6.96 (± 0.78) ^c	26	23.27 (± 2.82) ^b	26	283.46 (± 44.59) ^b
Diseased	26	2.15 (± 0.21) ^a	26	4.31 (± 1.50) ^a	26	48.20 (± 7.38) ^a
Healed	26	3.23 (± 0.27) ^b	26	4.15 (± 1.16) ^a	26	52.37 (± 8.55) ^a
August						
Healthy	25	20.88 (± 2.20) ^b	25	38.20 (± 3.80) ^b	9	497.23 (± 118.70) ^b
Diseased	25	4.72 (± 0.71) ^a	25	7.28 (± 2.19) ^a	10	114.90 (± 30.06) ^a
Healed	22	16.64 (± 2.14) ^b	22	43.73 (± 7.33) ^b	7	552.25 (± 127.74) ^b
October						
Healthy	23	3.22 (± 0.39) ^a	23	3.43 (± 0.23) ^a	23	51.45 (± 4.03) ^a
Diseased	21	3.38 (± 0.22) ^a	21	3.62 (± 0.26) ^a	21	51.28 (± 3.82) ^a
Healed	22	3.41 (± 0.31) ^a	22	3.18 (± 0.23) ^a	22	52.61 (± 3.07) ^a

n – number of observations, values – mean and standard errors (SE) – followed by the same index are not statistically different (multiple comparisons, $P > 0.05$) between treatments

ences between the characteristics under study were recorded again. In this case, the lesion data from the healthy trees differed from the others (Table 2)

and differences in the lesion width were found between all the treatments. The mean lesion surface area of the healthy material was 283 mm², in con-

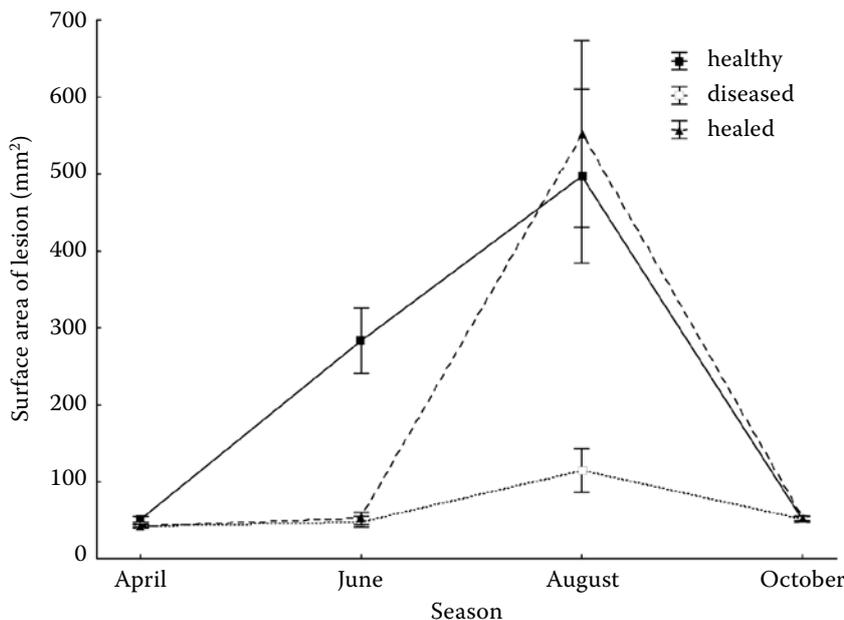


Fig. 1. Effect of health (natural infection of *P. alni*) and season on extent of artificial infection of *P. alni* in black alder (mean surface area of lesions, vertical bars represent standard errors of means)

Table 3. The correlations between parameters of lesions caused by *P. alni* on *A. glutinosa* branches (lesion width, length and area after one week's incubation) and branch section diameter in particular inoculation tests, ordered by date

Date of test	Branch section diameter (mm) (mean ± SE)	Width	Length	Surface area
July 2009	25.41 ± 1.06	-0.23	0.06	-
August 2009	27.93 ± 1.11	-0.05	-0.28	0.28
October 2009	21.02 ± 0.52	0.23	-0.11	0.23
April 2010	23.11 ± 0.51	0.41	0.18	0.35
June 2010	23.38 ± 1.07	0.17	0.35	0.46

values – mean and standard errors (SE), bold – Spearman's correlation coefficient, statistically significant correlations

trast to 48 and 52 mm² for the diseased and healed material, respectively. No differences were found in April 2010 and October 2009, when the extent of the lesions was generally limited (Table 2). The values of the mean lesion surface area ranged between 42 and 53 mm² in all the treatments and on both dates.

The year season of sampling was also found to be critical for the enlargement of necrotic areas. Statistically significant differences were found for all the lesion measurements in August 2009 in comparison with the other screening dates in the healed and healthy material (with the slight exception of the healthy material sampled in July, when the observed values of the lesion length and surface area were not different from those in August). Concerning the diseased material, statistically lower values for the width, length and surface area of the lesions were found in April and July, and the highest value was found in August (Table 2, Fig. 1).

The evaluation of the potential effect of the branch section diameter on lesion development showed a very weak relationship between the diameter of the tested branch section and the lesion development after one week of inoculation. The values of Spearman's correlation coefficient between the parameters of lesion width, length and area and the branch section diameters were not generally significant (Table 3). Only the lesion width and area in April 2010 and the lesion area in June 2010 positively correlated with the branch section diameters, but the observed correlations were weak.

DISCUSSION

An artificial inoculation method was used to study the factors affecting the development of lesions of *Phytophthora alni* ssp. *alni* in *Alnus glutinosa*.

The year season when samples were collected and the presence of naturally occurring *P. alni* infections were found to be important factors that affected the outcomes of artificial infection experiments. Therefore, these factors can be considered as potentially confounding the results of resistance screening programmes based on infection experiments using natural plant material.

A significant factor affecting the lesion growth was the initial health state of the material collected for these experiments, as the possible presence of naturally occurring infections of *P. alni* had to be taken into account. It is known that a common response to infection with the necrogenic pathogen in plants is the development of systemic resistance to a subsequent attack of the pathogen (RYALS et al. 1994). This induced resistance results in a long-lasting, broad-spectrum resistance in the remaining non-infected tissues, which could explain the observed differences in the lesion characteristics on the segments of branches taken from the acutely infected tree. However, the observed induced resistance in alder against *P. alni* seems to be ephemeral and declines once the natural infection subsides. It is very likely that small, hidden naturally occurred *P. alni* infections in the roots and collars of a tree could significantly affect the outcomes of infection tests performed using these affected trees. Thus, extreme caution is recommended in resistance screening programmes when using tissues of trees originating from affected alder stands.

A second important factor affecting the outcomes of infection tests was the season. The observed longitudinal extension rates of the necrotic tissue on the healthy sections of branches were the highest in July and August and the lowest in April. These results were similar to the report by BRASIER and KIRK (2001), although the range of rates we observed was slightly different. The high seasonal variability of al-

der susceptibility to *P. alni* ssp. *alni* infection, as described in this paper, is in agreement with seasonal changes in the lesion development reported for several other *Phytophthora* diseases, such as those of alder, oaks, common beech and other tree species (MATHERON, MIRCETICH 1985; ROBIN et al. 1994; BROWNE, MIRCETICH 1996; BRASIER, KIRK 2001; LUQUE et al. 2002; MORALEJO et al. 2009; HOLUB et al. 2010). The main cause of seasonal differences in host susceptibility is not well known. Differences in the lesion growth are sometimes explained by temperature considered as a limiting factor, which is positively correlated with the lesion development (SHEARER et al. 1987; LUQUE et al. 2002). In our case, the effect of temperature on the growth of the pathogen can be excluded because our trials were performed under standardized conditions. Differences are more likely explained by the host physiology. The greatest susceptibility occurred during the active growth periods when the trees were in full leaf; in contrast, the pathogen incidence was strongly suppressed in October and April, when the trees were dormant or just at the beginning of the new growing season. There could also be a relation with water status changes in the course of the year, as it has been shown that there is a correlation between the tissue water content and the length of *Phytophthora* lesions (e.g. TIPPETT et al. 1987; MARÇAIS et al. 1993; ROBIN et al. 1994).

Potential effects of the social status of the tested individual stems (as determined by their position in the multi-trunked tree), duration of the infection experiments and the thickness of branch sections used in the tests can likely be considered as negligible. Firstly, we found no difference in the development of lesions in the sections of branches collected from different parts of a multi-trunked tree; however, we do not exclude a possible effect of the social status. WOODRUM et al. (2003) obtained samples only from branches exposed to the sun in an attempt to reduce the possible effect of microhabitat on the mechanical properties of five *Acer* species. Secondly, the lesion dimensions increased almost linearly on the alder branches during the three weeks following the inoculation of the pathogen. In contrast, ROBIN and DESPREZ-LOUSTAU (1998) observed that the longitudinal lesion development on oak stems inoculated with *Phytophthora cinnamomi* was quite rapid in the first two weeks and then slowed down. However, with regard to the ascertained high growth rate of *P. alni* in our trials, the incubation period of one week is deemed to be sufficient for any subsequent screening programme. Thirdly, the thickness of the branch sections used in

our experiments was not found to affect the results after one-week duration of the experiment.

CONCLUSIONS

Our results confirm the strong influence of the year season of sampling and also of the presence of naturally occurring *P. alni* infections on artificial infection experiments carried out using field-collected black alder material. Although we could not show that the other tested factors had any important effect on the results (e.g. social status of the stem, duration of the experiment, diameter of the tested segments), it nevertheless seems appropriate to attempt to reduce any possible sources of variation to a minimum when sampling black alder populations for material to be used in screening for resistance to *P. alni* infection.

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